Sangamo BioSciences, Inc.

# Using Zinc-Finger Proteins in DNA Diagnostics

*In 1995, available medical diagnostics technology offered no simple, inexpensive means for the early detection of disease; for example, available tests could detect cancer only after a tumor appeared. Sangamo BioSciences, Inc. planned to develop a rapid, sensitive, and inexpensive approach to detect low-level gene mutations in DNA for DNA-based diagnosis. Sangamo therefore submitted a proposal to the Advanced Technology Program (ATP) and received funding for a three-year project under a 1995 focused program, "Tools for DNA Diagnostics." Sangamo proposed an innovative and technically challenging strategy to custom design highly specific engineered enzymes that will recognize genetic mutations with unique zinc-finger proteins (ZFPs). Sangamo believed they could engineer ZFPs to bind to specific DNA sequences of interest to detect cell disease in any human gene. If successful, genetic-based testing would yield susceptibility information that would lead to early treatment. The company targeted three areas: cancer, genetic disease, and pathogens that cause infectious disease. Technical risks included selecting and engineering the right proteins that bind to DNA sites with high affinity and in a sequence-specific manner, which had never been done. The proteins also needed to fold into specific patterns in order to make contact with the DNA sequences.*

*Sangamo accomplished much of its original technical goals in diagnostics by developing ZFPs and proteins for detecting Alzheimer's disease and infectious diseases such as hepatitis B and HIV. However, the company was unable to find partners to continue funding diagnostics development, because investors felt that therapeutic treatments would yield a higher return on investment. The work on this ATP-funded project did effectively position Sangamo to pursue ZFP applications for therapeutic treatment, which became the market direction. In 1997, Sangamo began to pursue a therapeutics approach simultaneously with its ATP-funded diagnostics work; therapeutics became the company's primary focus after this project ended in July 1998. Sangamo researchers published their results widely and were awarded three patents for the pioneering advances in ZFP technology in diagnostics. The company raised $60 million from an initial public offering in 2000 and acquired a competitor in 2001. As of 2005, Sangamo was collaborating with numerous pharmaceutical partners in developing ZFPs for therapeutics for peripheral artery disease, HIV, diabetic neuropathy, and other diseases. Although the development of ZFP technology still involves challenges, if any one of Sangamo's therapeutic ZFPs succeeds and reaches commercialization, it can save thousands of lives.*

**COMPOSITE PERFORMANCE SCORE**
(based on a four star rating)
* *

**Research and data for Status Report 95-08-0016 were collected during February – March 2005.**

## Diseases Are Reflected in a Patient's DNA

Human DNA is composed of two strands of linked nucleotides with one of the four bases (adenine, A; thymine, T; guanine, G; and cytosine, C), with 3 billion base pairs altogether, called the genome. The two strands are complementary; the paired nucleotides hold the two strands together in a long double helix. A unique sequence of the four chemical bases (called nucleotides) determines an individual's genetic code.

DNA provides instructions for the cell's activity. Transcription, a key process in cellular health, copies information via enzymes from DNA into new strands

of messenger ribonucleic acid (mRNA) in order to do the work of the cell. The mRNA instructs the cell to manufacture specific proteins. Transcription takes place in the cell nucleus; the mRNA moves from the nucleus to the protein production site in ribosomes in the cytoplasm. A ribosome is a subcellular structure that makes proteins for the cell; it interacts with mRNA and joins amino acid units (the building blocks of proteins) together as determined by the genetic code. Many diseases result in overproduction or underproduction of proteins within the cells. If scientists could manipulate the transcription process, they could use it to treat disease.

In order to correct the transcription process, researchers must first recognize the disease. Many diseases can be identified by detecting a gene mutation through DNA analysis. In 1995, however, existing technologies offered no simple, inexpensive way to find mutations in genes. Existing DNA tests were labor intensive, slow, and expensive, and they required highly trained staff.

One disease that is caused by gene mutation is sickle cell disease, the most common inherited blood disorder in the United States, which affects about 1 in 500 African Americans, or 72,000 people. About 8 percent of African Americans are carriers. Sickle cell disease is caused by a mutation of a single letter in the genome that codes for hemoglobin, the iron-containing pigment of the red blood cells that carries oxygen. In patients with sickle cell disease, the abnormal red blood cells are crescent shaped like sickles. They attach to one another, forming long rods that can clog blood vessels, deprive organs of oxygenated blood, and cause crippling pain. Because sickle cell disease is caused by a single defective gene, DNA technologies hold promise for detecting and treating it. If scientists could detect and repair the base pair change, without making any other DNA changes, the patient with sickle cell disease could be cured.

Another disease that could benefit from genetic diagnostics and therapy is peripheral artery disease. Three million Americans suffer from heart failure, with 400,000 new cases each year and more than 39,000 deaths. If early peripheral artery disease could be detected, many lives could be saved.

## Zinc-Finger Proteins Could Enable Early Diagnosis

Dr. S. Chandrasegaran of Johns Hopkins University had developed restriction enzymes containing DNA-binding proteins, called zinc fingers. A restriction enzyme is a protein that recognizes specific, short nucleotide sequences and cuts DNA at those sites. A zinc finger is a protein motif that interacts with DNA and RNA. The finger-like fold is created by binding specific amino acids in the protein to a zinc atom. Cutting the DNA in specific disease-related sites could lead to correction and improvement.

***Existing DNA tests were labor intensive, slow, and expensive, and they required highly trained staff.***

Sangamo BioSciences, Inc. was a start-up biotechnology company dedicated to developing custom restriction enzymes and DNA-binding proteins. The company was interested in developing highly specific zinc-finger proteins (ZFPs) and custom restriction enzymes that would bind to DNA sequences that are important to the human diagnostics field. Sangamo, however, faced technical risks in designing or selecting proteins that bind to DNA sites in a sequence-specific manner, which had never been done. Once the proteins had been formed, they needed to fold into the right patterns in order to make contact with targeted DNA sequences in order to alter cell function. Because of these risks, Sangamo was unable to raise adequate funds. ATP awarded cost-shared funding for three years as part of a 1995 focused program, “Tools for DNA Diagnostics.”

Sangamo proposed to develop inexpensive, highly specific proteins for detecting known human genetic mutations by using ZFPs that bind to specific DNA sequences affected by disease and efficiently test these binding proteins against diseases. The first screening targets would be cancer, infectious diseases, and genetic diseases, such as sickle cell disease. Simple, inexpensive, and sensitive methods to distinguish subtle DNA changes could facilitate early detection and treatment.

ZFPs attach to the DNA at specific sites in the DNA sequence (see Figure 1). They can detect individual gene mutations, which affect the cell's function (production of specific proteins may be activated or repressed).

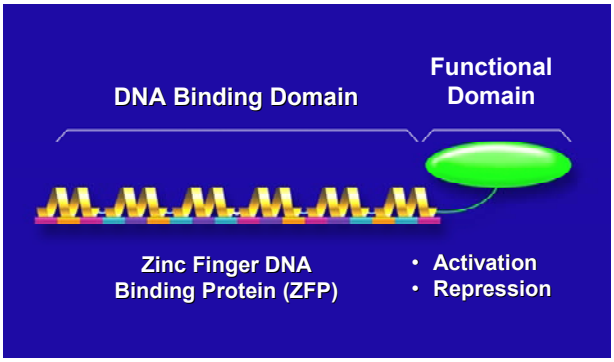


Figure 1. Sangamo engineers novel transcription factors to mimic the natural mode of gene regulation. They customize ZFPs to recognize a DNA sequence close to or within a gene of choice. The ZFP can change the function of the target gene (activate or repress protein production).

Each "finger" structure of a ZFP is a small modular unit that recognizes and binds to three base pairs of DNA. These modules can be joined together to bind longer DNA sequences (see Figure 2). As shown in the illustration on the left, a three-finger ZFP (in green) binds to a nine-base-pair-long sequence of DNA (in blue). Each finger contains a zinc atom (pink) that maintains its structure; one particular region of the finger, called the alpha helix (depicted in yellow on the first finger), makes contact with the DNA. In the illustration on the right, a closer view of the helix shows the four key amino acid residues that make contact with the DNA. If different residues are substituted in these positions, then a different sequence of DNA will be recognized and bound. Sangamo has done extensive work to determine which combinations of residues at these positions enable recognition and binding to which DNA bases.

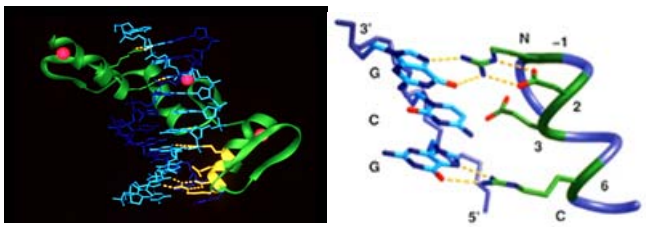


Figure 2. Left: A three-finger ZFP (in green) binds to a nine-base-pair-long sequence of DNA (in blue). A zinc atom (pink) maintains its structure; the alpha helix (yellow on the first finger) makes contact with the DNA. Right: A closer view of the four key amino acid residues that make contact with the DNA helix.

The molecular diagnostics and genetic testing market was expected to grow from $200 million in 1994 to $1 billion by 2000. As of 1995, 25 percent of genetic testing was being done in university labs, 25 percent in community hospitals, and 32 percent in private labs. If Sangamo were successful, markets would consider zinc-finger technology a viable, lower cost alternative to existing technologies such as complete DNA sequencing, which was slow and costly. Developing automation capabilities would be key in achieving labor, time, and cost savings. In its future work, Sangamo also hoped to develop reagents or reagent-based diagnostic kits, therapeutic applications, and veterinary and agricultural products.

## Sangamo Collaborates with Top Researchers

The project had challenging technical goals. Engineering proteins that bind to DNA in a sequence-specific manner had never been done and building these proteins to be specific enough to be used as diagnostics had also never been achieved. Sangamo's technical goals were to:

- Identify and modify restriction enzymes that affect specific DNA sequences
- Engineer DNA-binding ZFPs
- Demonstrate DNA mismatches using ZFPs between healthy specimens and unknown samples
- Develop a complete detection system for known disease mutations using ZFPs, which could be automated
- Develop an enzyme for detecting unknown mutations, called MutS chimeric restriction enzyme

To address these goals, Sangamo formed partnerships and licensed intellectual property from top researchers at Johns Hopkins University in Baltimore, MD, the Laboratory of Molecular Biology in London, Scripps Research Institute in La Jolla, CA, and the Massachusetts Institute of Technology in Cambridge, MA. These collaborations continued throughout the ATP-funded project.

## Sangamo Analyzes Three Areas for Gene Regulation

Sangamo started by analyzing a gene, called the ApoE gene, using zinc fingers to look for known mutations associated with increased risk for Alzheimer's and cardiovascular disease. They constructed two zinc-finger restriction enzymes that were designed to bind DNA sequences including the E4 or E3 alleles (variants) of the gene. Patients with the E4 mutation had increased risk for Alzheimer's. Sangamo would engineer two ZFPs to recognize and discriminate between the two alleles. Then they would purify the ZFPs.

A second area of development was building ZFP IgG1 (antibody) fusion proteins to develop a detection method that relied on binding of the fusion protein to DNA. Sangamo was able to construct two proteins with ZFPs that fused to the IgG1 protein; however, ultimately, this part of the project failed, because these fusion proteins were not active.

Sangamo developed a procedure to purify the MutS chimeric restriction enzyme from E. coli. MutS detects mutations in DNA, which reflect disease. Sangamo's idea was to use this procedure to find differences between two DNA sequences (a healthy sample versus an unknown sample). They would put the two DNA sequences together, and MutS would recognize the difference. However, this process was too difficult, and Sangamo was never able to make it work. The company hoped to expand zinc-finger technology to detect other genetic mutations. The goal for the third year of the project was to extend development work and to develop an automated detection system.

## Project Proves the ZFP Concepts

Sangamo added a team of scientists to verify ZFPs for E3 and E4 alleles on the ApoE gene, as an indicator of late-onset Alzheimer's. They used the model for detecting the E4 allele to design new ZFPs to detect infectious diseases.

The company achieved a significant percentage of their original technical goals. The concepts remained viable for Alzheimer's and infectious disease diagnostic testing. Existing DNA diagnostic tests required 8 to 10 hours to generate results; Sangamo had achieved a 2-hour manual turnaround, with a target of 30 to 45 minutes. The biggest problem was in the sample amplification (copying) stage; Sangamo would later find commercial partners from which to license amplification technology and to automate the processes.

***Sangamo proposed to develop inexpensive, highly specific proteins for detecting known human genetic mutations by using zinc-finger proteins that bind to specific DNA sequences affected by disease.***

By the end of the project in 1998, Sangamo had engineered ZFPs, had purified them, and had developed assays to measure affinity. The company was able to build fusion proteins that would preferentially bind to the E3 and E4 alleles of the ApoE gene. Sangamo published its results in academic journals and was awarded three patents for work directly resulting from this project. Sangamo approached DNA diagnostics companies to continue development toward automation and commercialization, but Sangamo was unsuccessful because the companies were more interested in funding therapeutics applications, which anticipated higher return on investment. The pioneering research conducted with ATP funding had proved the viability of ZFP technology, but Sangamo was unable to find funding to commercialize ZFP diagnostics.

## Sangamo Changes Focus to Therapeutics

In response to market demand, Sangamo shifted from diagnostics to therapeutics. According to Dr. Casey Case, Vice President of Research Operations at Sangamo, this ATP-funded project "was a critical early step in the development of our technology and the establishment of the company. We made a strategic decision to move out of the diagnostics business, but many of the things we learned about [ZFP] engineering have been applied to our current work. Our emphasis now is on human therapeutics…. All of our therapies use engineered ZFPs. We learned a lot about…ZFP engineering during the ATP-funded research." This project represented the beginning of commercial applications of ZFP engineering outside an academic

environment, he said. The knowledge gained during the diagnostics project regarding engineering ZFPs with high specificity, purity, and function spun off new research and applications in therapeutics.

Toward the end of the ATP-funded project, Sangamo raised $7 million from private investors, which they dedicated to therapeutics development. Therapeutics had higher potential rewards, if Sangamo could achieve a single success. The company started a second ATP-funded project focused on therapeutics in 1997 ("Development of Novel DNA Binding Proteins as Antiviral Therapeutics," 96-01-0315).

## ZFP-Based Therapeutics Prove Successful for Sangamo

Based on its ZFP therapeutics technology, Sangamo received $60 million from an initial public offering in 2000. In 2001, Sangamo acquired Gendaq, a private biotechnology firm based in London, which focused on engineering ZFPs. As of 2005, Sangamo was performing collaborative research with a number of pharmaceutical companies in pursuit of treatments for diabetic neuropathy, peripheral artery disease, diabetes, HIV, congestive heart failure, cancer, Hepatitis B, sickle cell anemia, Severe Combined Immunodeficiency Disease (SCID or “bubble boy” disease), and others.

***Sangamo had engineered zinc-finger proteins, had purified them, and had developed assays to measure affinity.***

In addition to its efforts to cure diseases through ZFP therapeutics, Sangamo has identified other goals. For example, the company is engineering cells to produce useful proteins such as growth hormones and antibodies. They are developing bioengineering methods for manufacturing. And, in 2004, they began another ATP-funded project to use ZFPs to regulate genes in plants, potentially leading to new crops for optimized yield or enhanced nutritional properties (“Targeted Activation of Multiple Genes in Plants Using a Single Engineered Transcription Factor,” 00-00-5559).

## Conclusion

Sangamo BioSciences, Inc. used this 1995 ATP-funded focused project in “DNA Diagnostics” to pioneer zinc-finger-protein (ZFP) engineering. They used ZFPs to detect specific gene mutations in DNA for early diagnostics. Although Sangamo shifted its focus away from DNA diagnostics to DNA therapeutics to meet market demands after the ATP-funded project, the technical skills and the knowledge of ZFP engineering gained during this project laid the foundation for much of the company’s later work in therapeutics and plant genetics. Sangamo published its findings and received three patents for work directly resulting from this project. In addition, later projects in ZFPs for human therapeutics and plant engineering could not have been done without this earlier success. Sangamo conducted an initial public offering in 2000 and acquired a competitor, Gendaq, in 2001. As of 2005, the company was performing collaborative and contract research with numerous pharmaceutical and research institutions in pursuit of cures treatments for diabetic neuropathy, peripheral artery disease, diabetes, and HIV.

# PROJECT HIGHLIGHTS
## Sangamo BioSciences, Inc.

**Project Title:** Using Zinc-Finger Proteins in DNA Diagnostics (Generation and Development of Novel Nucleic Acid Binding Proteins and Their Use as DNA Diagnostics)

**Project:** To develop a versatile and robust means of generating protein-based agents capable of binding and cleaving DNA at any predetermined site as the basis of a mutation-detection system intended for diagnosing genetic and pathogenic human diseases, as well as for human therapeutics, research reagents, and veterinary/agricultural products.

**Duration:** 8/1/1995 - 7/31/1998
**ATP Number:** 95-08-0016

**Funding (in thousands):**

| | | |
|---|---|---|
| ATP Final Cost | $1,999 | 89.4% |
| Participant Final Cost | 236 | 10.6% |
| Total | $2,235 | |

**Accomplishments:** Sangamo BioSciences, Inc. accomplished a significant percentage of its original technical goals for developing DNA diagnostics relying on zinc-finger proteins (ZFPs). Sangamo's advances achieved in this project are listed below:

- Sangamo engineered ZFPs and purified them.
- They developed assays to measure ZFPs' affinity for binding to the intended DNA target.
- They were able to build proteins that would preferentially bind to the E3 and E4 alleles of the ApoE gene, which indicates susceptibility to Alzheimer's disease.
- They used the ApoE gene model to design new ZFPs to detect infectious diseases.

Sangamo was awarded three patents from this ATP-funded technology:

- "Methods of using randomized libraries of zinc finger proteins for the identification of gene function" (No. 6,503,717: filed December 6, 2000; granted January 7, 2003)
- "Gene identification" (No. 6,780,590: filed August 28, 2001; granted August 24, 2004)
- "Selection of sites for targeting by zinc finger proteins and methods of designing zinc finger proteins to bind to preselected sites" (No. 6,785,613: filed March 28, 2002; granted August 31, 2004)

**Commercialization Status:** Although no direct commercial products have yet resulted from ZFP diagnostic technologies, Sangamo performs collaborative research with several pharmaceutical companies. The technology holds significant promise. Sangamo has raised more than $100 million in funding since 1995.

**Outlook:** The outlook for ZFPs is good, but their development still poses challenges in clinical applications. Although the potential benefits from ZFP-based therapeutics are enormous, the risk is still high. Sangamo is building on the foundation technology from this project in seeking treatments for diabetic neuropathy, peripheral artery disease, diabetes, HIV, sickle cell anemia, and other monogenic diseases.

**Composite Performance Score: * ***

**Number of Employees:** 3 employees at project start, 38 as of August 1998, 50 as of March 2005.

**Focused Program:** Tools for DNA Diagnostics, 1995

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**Subcontractor:**

- Johns Hopkins University
  Baltimore, MD

# PROJECT HIGHLIGHTS
## Sangamo BioSciences, Inc.

**Publications:** Sangamo researchers disseminated their findings and received public attention through the following publications:


- Liu, Q., Z. Xia, X. Zhong, and C. C. Case. "Validated Zinc Finger Protein Designs for all 16 GNN DNA Triplet Targets." *J Biol Chem*, February 8, 2002, 277 (6), pp. 3850-6. Erratum in: *J Biol Chem*, 277 (16), p. 14350, April 19, 2002.

- Urnov, F. D., E. J. Rebar, A. Reik, and P. P. Pandolfi. "Designed Transcription Factors as Structural, Functional and Therapeutic Probes of Chromatin in Vivo." Fourth in review series on chromatin dynamics. *EMBO Rep*, 3 (7), pp. 610-5, July 2002.

- Snowden, A. W., P. D. Gregory, C. C. Case, and C. O. Pabo. "Gene-Specific Targeting of H3K9 Methylation Is Sufficient for Initiating Repression in Vivo." *Curr Biol*, 12 (24), pp. 2159-66, December 23, 2002.

- Jamieson, A. C., J. C. Miller, and C. O. Pabo. "Drug Discovery with Engineered Zinc-Finger Proteins." *Nature Reviews: Drug Discovery*, 2(5), pp. 361-8, Review, May 2003.

- Tan, S., D. Guschin, A. Davalos, Y. L. Lee, A. W. Snowden, Y. Jouvenot, H. S. Zhang, K. Howes, A. R. McNamara, A. Lai, C. Ullman, L. Reynolds, M. Moore, M. Isalan, L. P. Berg, B. Campos, H. Qi, S. K. Spratt, C. C. Case, C. O. Pabo, J. Campisi, and P. D. Gregory. "Zinc-Finger Protein-Targeted Gene Regulation: Genomewide Single-Gene Specificity." *Proceedings of the National Academy of Sciences* 14, 100 (21), pp. 11997-2002, October 14, 2003.

- Weiss, Rick. "Technique to Fix DNA Flaws Is Tested." *Washington Post*, p. A2, April 4, 2005.

- Flores, Graciela. "Zinc Finger Nucleases Correct Genes." *The Scientist*, April 4, 2005.